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Ligation-based DNA Diagnostics

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Summary

A number of novel gene detection techniques all revolve around the ligation of synthetic nucleic acid probes. In such ligase-assisted gene detection reactions, specific DNA or RNA sequences are investigated by using them as guides for the covalent joining of pairs of probe molecules. The probes are designed to hybridize immediately next to each other on the target nucleic acid strand. Demonstration of ligated probes results in highly specific detection of and efficient distinction between similar sequence variants under standard reaction conditions. Accordingly, the principle has been applied in automated genetic screening procedures. Ligation reactions are also integral to a number of amplification procedures and they will be of value in an expanding range of genetic analyses.

Introduction

In nature, DNA ligases serve the important function of sealing interrupted DNA strands during DNA replication and repair and in genetic recombination. Once enzymes that can join DNA strands had been isolated and biochemically characterized, they proved to be of crucial importance for constructing recombinant DNA molecules, assembled from different DNA segments and propagated and expressed in new organisms.

More recently, ligases have been used in combination with short, synthetic DNA molecules to permit the construction of an intriguing range of molecular structures. Synthetic oligonucleotides can be designed to include the four natural bases in any order. Their sequence may be selected so that they will base-pair with other DNA molecules in a predetermined manner, with, as usual, approximately 10 base pairs or 3.4 nm per turn of the double helix. Molecular complexes established by hybridization between such synthetic DNA molecules may be covalently joined through the agency of a ligase. In this manner, DNA molecules with topologies not observed in nature can be created using a molecular Meccano. For instance, Seeman and coworkers have constructed DNA molecules with the topology of cubes. These molecules are composed of six circular oligonucleotides, engaged in 12 duplex segments that correspond to the edges of the cube[1,2]. This type of DNA structure could serve as a molecular scaffold, suitable for positioning and manipulating other molecular elements with an unprecedented combination of spatial freedom and resolution. It has been proposed that DNA complexes constructed in a similar manner could serve as computer chips[3], but they have not yet been put to routine use in this capacity. By contrast, ligation of synthetic oligonucleotide probes has come to play an increasing role in DNA diagnostic investigations. Before going on to describe the particulars of such ligase-assisted assays, I will briefly discuss some technicalities of ligation reactions.

Mechanisms and Methods of Joining Nucleic Acid Strands

DNA ligases join DNA chains by converting the energy of a pyrophosphate linkage in a nucleotide cofactor into a phosphodiester bond between a 5′ phosphate group and a 3′ hydroxyl on two adjacent nucleic acid molecules[4,5]. In a first reaction step, lysine residues of the ligases form a phosphoamide linkage with AMP, derived from the cofactors NAD or ATP. The stored energy is then used to generate a phosphodiester linkage by first transferring the AMP group from the enzyme to the 5′ phosphate group. Next, a nearby 3′ hydroxyl is subjected to nucleophile attack, resulting in the joining of two polynucleotide strands.

The DNA ligase from bacteriophage T4 can join both DNA and RNA strands, and it can use either DNA or RNA templates to align the strands to be ligated. Reactions only involving DNA strands proceed with greater efficiency, however. Also DNA duplexes with base-paired (blunt) ends can be joined by the phage ligase. Recently, a number of ligases have been isolated from thermophile organisms[6-8]. These enzymes operate at high reaction temperatures and survive conditions that denature DNA. They also exhibit a reduced tendency to join double-stranded DNA molecules with base-paired or with short complementary ends. These properties result in increased specificity of detection and convenience in many analytical reactions.

There are also chemical means for joining polynucleotide chains through a phosphodiester linkage in a template-dependent manner[9,10]. Substrate requirements in these reactions may differ from those of enzyme-catalyzed reactions. For example, cyanogen bromide preferentially joins strands with a 5′ hydroxyl and a 3′ phosphate group, respectively.

Efficient means of ligating nucleic acid molecules, in combination with designer oligonucleotides, now open the door to a wide selection of gene detection assays.

Ligase-mediated Gene Detection

Ligase-assisted analytical reactions depend on demonstrating that two or more probe segments have been joined to form a continuous molecule by a DNA ligase. This reaction in turn requires that the probes hybridize in immediately adjacent positions on a target nucleic acid sequence (Fig. 1)[11]. The strategy contrasts with more conventional gene detection schemes where the hybridization between a detectable probe and a target sequence is visualized after removal of free probe molecules. Ligase-based gene detection reactions exhibit a number of valuable properties:

Fig. 1. A schematic description of the oligonucleotide ligation reaction. Two oligonucleotide probes, shown in black, hybridize in immediately adjacent positions on a target nucleotide sequence. The position where the probes become joined by ligation is circled.

1) The detection reactions are inherently very specific: ligation depends on the coincidence of two independent probe sequences on a complementary molecule. This is unlikely to occur in the absence of the appropriate target molecule. For this reason assays perform adequately under a wide range of experimental conditions and reaction conditions may be standardized.

2) The ligation reaction can be employed to assess the accuracy of hybridization at positions immediately surrounding the junction between the two probes. Due to the substrate requirements of ligases, terminally mismatched probes are ligated at a substantially reduced rate. In this manner, allelic sequence variants can be conveniently distinguished.

3) The act of ligation creates a covalent molecule, not previously present in the reaction mixture. This circumstance can be employed by taking advantage of the increased hybridization stability upon ligation. As described below, probes that have ligated can also be recruited as templates in exponential amplification reactions. Ligated molecules may also acquire other properties that can be employed to ensure efficient detection, including insensitivity to exonucleases or the covalent coupling of a variety of detectable groups to a support.

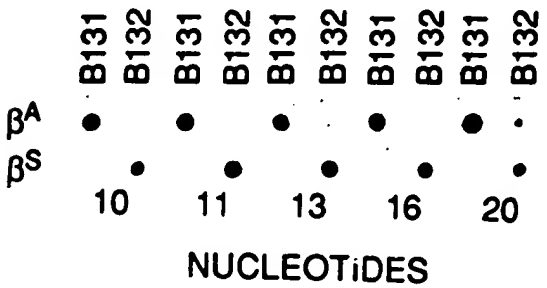

Fig. 2. Relative length independence of oligonucleotide probes used to distinguish closely similar DNA sequences. Two twenty-nucleotide-long probes, one (B131) specific for the normal variant of the β globin gene (β^A) and one (B132) specific for the sickle cell mutation (β^S), were compared for their ability to ligate, under identical conditions, to adjacent oligonucleotides of the indicated sizes. The two allele-specific probes differ at the last nucleotide position. Ligation products were identified by autoradiography as described[11]. The results demonstrate that the assay is relatively insensitive to the stability of hybridization, in that allele-specific reagents correctly identify the appropriate sequence variant under identical reaction conditions using a wide range of oligonucleotide sizes.

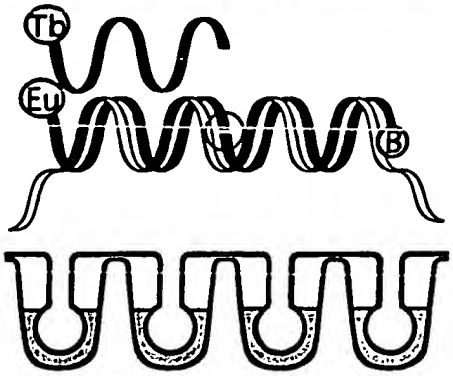

Fig. 3. Dual-color ligation analysis of DNA sequence variants. Of the three oligonucleotides used (shown in black) one oligonucleotide is labelled with europium ions (Eu) and is specific for one sequence variant. Another oligonucleotide, labelled with terbium ions (Tb), is of similar sequence but it is specific for another sequence variant. These two probes differ in sequence such that each of two possible target sequence variants present in a sample can only support ligation of one of the lanthanide ion-labelled oligonucleotides to the downstream oligonucleotide. This third oligonucleotide, along with any oligonucleotide ligated to it, may be bound to an avidin-coated support through a 3′ biotin group (B), followed by detection of the probes by fluorescence analysis. Also shown is a set of four prongs from a 96-pronged manifold, introduced into wells of a microtiter plate to collect biotinylated ligation products before a visualization step.

The Oligonucleotide Ligation Assay (OLA)

The ligation of probe molecules can be investigated using a simple detection format where one of the two oligonucleotides carries a detectable group. This could be a radioisotope or a fluorophore. The other probe can be retrieved through an affinity ligand such as biotin, or it may be bound to a solid support before the ligation step. After the reaction, joined probe molecules can be collected and quantitated. T4 DNA ligase-assisted reaction conditions may be defined that support the joining of all correctly base-paired pairs of probes but any mismatch, if located at the junction of the two oligonucleotide probes, severely compromises ligation efficiency[11]. Single mismatches, located up to three nucleotide positions upstream or downstream of the junction between the two probes, can be distinguished (U. L., unpublished results). The enzyme-assisted distinction between target sequences, differing in single nucleotide positions, proceeds satisfactorily under identical conditions using oligonucleotide probes, varying from 10 to 20 nucleotides in length (Fig. 2). Furthermore, single copy gene sequences are detected with adequate specificity in total human genomic DNA at a temperature 25°C below the melting temperature of the probes[11]. These results attest to the specific nature of the ligation reaction and its suitability in standardized genetic assays. Wu and coworkers have also examined some of the properties of T4 DNA ligase-mediated detection reactions[12].

The ligation assay has been used in an automated genetic

screening format. In this procedure DNA segments, amplified by the polymerase chain reaction (PCR), are analyzed for the presence of DNA sequence variants associated with disease, or serving as genetic markers. As a consequence of target dependent ligation, a detectable group present on one of the probes becomes covalently connected to the other probe. This probe, in turn, is immobilized in a microtiter well before a visualization step[13,14]. More recently we have developed a highly efficient test format, using double-labelled probe sequences, specific for each of two sequence variants[15] (Fig. 3). As labels we use rare earth metal chelates of europium and terbium ions that can be sensitively detected, distinguished and quantitated by time-resolved fluorometry. In this manner, allelic sequence variants are distinguished or copy numbers of specific nucleic acid molecules can be quantitated and compared to an externally added control sequence. The detectable groups can be introduced as modified phosphoramidites during standard oligonucleotide synthesis. The expedience of this protocol is further enhanced by using a high-capacity manifold solid support to collect sets of 96 reaction products and transport these between incubations; this also reduces the risk of contamination and mix-up of samples[16].

Ligase-assisted Amplification Reactions

Ligation steps are integral parts of many amplification procedures based on the PCR principle. For example, in order to amplify an uncharacterized piece of DNA located next to a known nucleotide sequence, an oligonucleotide can be added by ligation to the remote end of the desired DNA fragment. This added sequence may then be used as a handle in the amplification reaction[17,18].

Target-dependent ligation reactions have been applied in a cyclical fashion, resulting in the exponential accumulation of ligation products, in a technique called the ligation amplification reaction[19], or ligase chain reaction[20,21] (Fig. 4). In this procedure not one but two pairs of probes are used, each pair representing one of the strands of the target DNA sequence under study. If the probe pairs hybridize to target sequences,

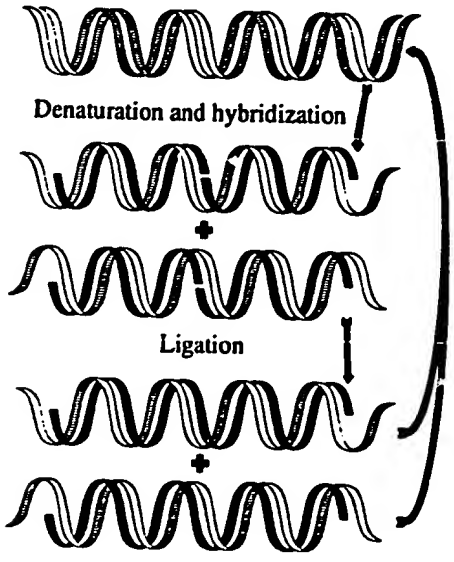

Fig. 4. The ligase chain reaction to detect specific sequences. A target DNA segment is denatured and four oligonucleotide probes (shown in black) hybridize in pairs on both strands of the target segment. After ligation, two copies of the original double stranded target segment have been generated and the cycle of denaturation, hybridization and ligation is reinitiated. In this manner, ligated probes accumulate exponentially.

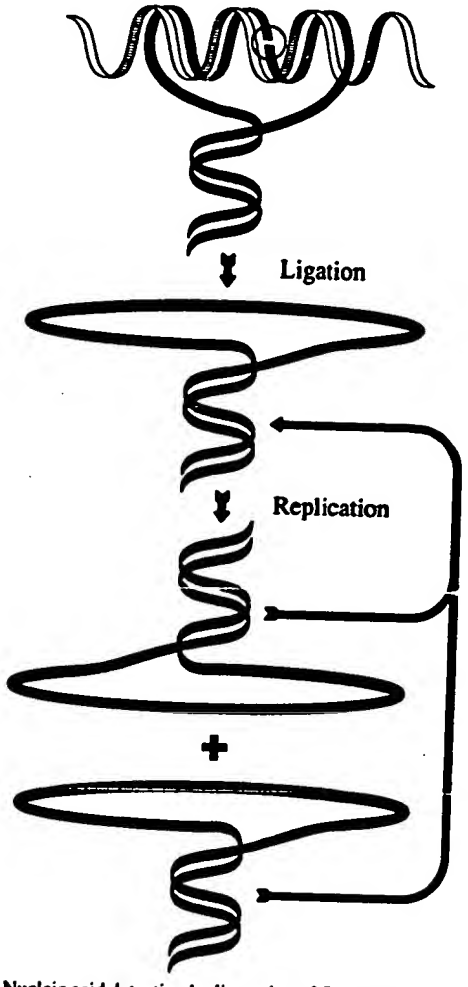

Fig. 5. Nucleic acid detection by ligase-based Q_β amplification. The enzyme Q_β replicase has the capacity to replicate exponentially RNA molecules exhibiting certain structural features. Two RNA molecules (shown in black), each having a target-complementary region, hybridize in juxtaposition on a target sequence. While the two probe molecules cannot be replicated, after ligation a molecule capable of exponential replication by the Q_β replicase is formed. The specificity of the assay is further enhanced by probe capture on, and release from, a solid support (not shown)[25].

they can be joined by ligation. When the samples are denatured again, rendering the molecules single-stranded, a new cycle of probe ligation is initiated. Probes that have been joined by ligation can serve as templates for the ligation of complementary oligonucleotide pairs in subsequent cycles of ligation. The use of a thermostable ligase permits stringent reaction conditions, increasing the specificity of detection. It also ensures that the enzyme survives the cyclical increase of the temperature required to denature DNA strands. An added benefit of this assay is that point mutations, located at the junction between the probe pairs, may be distinguished directly in genomic DNA. In a variation of the above strategy the members of the probe pairs hybridize some distance away from each other, requiring the addition of a few nucleotides by a polymerase before the ligase can join the probe pairs[22]; see also[23].

The highly specific, target-dependent ligation reaction can also be used to initiate exponential amplification by the enzyme Qβ replicase. This peculiar enzyme, derived from *E. coli* cells infected by bacteriophage Qβ, has the capacity to translate, as it were, certain RNA sequences into complementary RNA sequences. It then proceeds to copy both the original and the copy RNA strands at a fixed temperature, resulting in a rapid amplification reaction: in 30 minutes at a fixed temperature a 10^9-fold amplification results[24]. The amplification reaction is initiated in a target-dependent manner if two suitably configured RNA probes, with target-complementary sequences at one end each, are joined by a ligase. Only if the probe halves are joined by ligation can these subsequently be replicated autocatalytically by the Qβ replicase, resulting in a simple detection reaction that exhibits excellent specificity and sensitivity[25] (Fig. 5).

Future Applications for Ligation Reactions

Ligation reactions continue to be applied in new analytical contexts. Any desired primer for Sanger-type sequencing may be assembled from a library of all hexamer oligonucleotides by ligation[26]. In a novel approach to DNA sequence analysis, the complete sequence of a DNA segment of interest is deduced on the basis of information about which of all possible n-mer oligonucleotides are part of the fragment[27-29]. The analysis can be based on oligonucleotide hybridization, but this process may be enhanced by substituting a target-dependent ligation step (Charles Cantor, personal communication).

Alves and Carr have demonstrated the possibility of using the *in situ* ligation of synthetic oligonucleotide probes hybridizing to blots as a means of locating specific sequences among gel-separated human genomic DNA molecules[30]. In a similar manner, the specificity and increased stability of ligated probes could permit the use of synthetic oligonucleotide probes in *in situ* ligase-assisted analyses of sequences expressed as RNA molecules in tissue sections or of genes in metaphase chromosomes.

Simple but highly specific and sensitive ligase-assisted detection reactions, such as the Qβ amplification reaction referred to above, can be performed in unpurified patient samples. The required reagents could be provided in a stabilized, prealiquoted form[31]. By monitoring amplification in a simple colorimetric manner the assays are likely to prove sufficiently straightforward and inexpensive to be suitable for detection of infectious diseases without the benefit of a laboratory, eg. in a third-world setting.

Finally, future gene diagnostic investigations will have to face an increasing number of gene sequences for analysis in order to predict common polygenic diseases. Also in infectious diagnosis many different DNA sequences may have to be investigated in parallel in order to identify the infectious agent. DNA diagnostics are likely to undergo continuing development towards miniaturization, in a way analogous to that of electronics, with ever more samples analysed together and more assays performed in a single DNA sample. In these situations, ligase-assisted detection reactions hold promise as a robust analytic principle to build the analytic strategies on. In conclusion, ligase-based assays will offer simplified genetic assays in a wide range of DNA diagnostic situations.

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